Understanding type 1 diabetes through proteomics.

Inne Crèvecoeur[1], Saurabh Vig[1], Chantal Mathieu[1*] and Lut Overbergh[1*]

[1] Laboratory for Clinical and Experimental Endocrinology, KU Leuven, Leuven, Belgium

* Equal contribution

Corresponding author:

Dr. Lut Overbergh
Laboratory for Clinical and Experimental Endocrinology,
KU Leuven, O&N1 Herestraat 49 bus 902,
3000 Leuven, Belgium

E-mail: lut.overbergh@med.kuleuven.be
Tel. +32 16 37 74 66
Fax: +32-16-346035

Abstract

Introduction: Autoimmunity against pancreatic beta-cells leads to an absolute shortage of the hormone insulin, resulting in hyperglycemia and the onset of type 1 diabetes (T1D). Proteomic approaches have been used to elucidate the mechanisms of beta-cell dysfunction and death.

Areas covered: In the present review, we discuss new insights in the beta-cell proteome that have contributed to better insights in the role of the beta-cell in T1D. Techniques, such as 2D-DIGE and MALDI imaging, together with new approaches for sample preparation, including laser capture microdissection and immunopeptidomics, have resulted in novel mechanistic insights in the pathogenesis of T1D. We describe how proteomic studies in beta-cell lines as well as isolated islets from animal models and humans have discovered intracellular signaling pathways leading to beta-cell destruction, the generation of neo-antigens through post-translational modifications of beta-cell antigens as well as better biomarkers of disease progression.

Expert commentary: Proteomics has contributed to the discovery of beta-cell neo-autoantigen generation through posttranslational modifications, hybrid insulin peptide formation and the generation of defective ribosomal gene products. These concepts are revolutionizing our insights in the pathogenesis of T1D, acknowledging a central role for the beta-cell in its own destruction.

1. Introduction

Type 1 diabetes (T1D) is chronic autoimmune disease in which the immune system destroys the insulin-producing beta-cells, a process called insulitis [1]. A model explaining the progressive development of T1D in different phases was first described by George Eisenbarth in 1986 [2]. He proposed that the combination of genetic predisposition and unknown triggers initiate an autoimmune reaction against the beta-cells, leading to dysfunction and destruction. Whereas for a long time it was believed that the immune system was the only culprit in T1D, several investigators, amongst which Botazzo, launched the idea that the beta-cell may not be an innocent victim but actively participates in its own destruction, a theory that is widely accepted and proven nowadays [3-6].

A generally accepted hypothesis for development of T1D suggests that aspecific inflammation, caused for instance by a viral infection, could be the first trigger initiating T1D. Such inflammation can affect the beta-cells by activation of Nuclear factor-kappa-B (NF-κB) and Interferon regulatory factor 3 (IRF3), resulting in the release of pro-inflammatory cytokines, such as interferon-α (IFN-α) and IFN-β, which in turn will activate Signal transducer and activator of transcription 1 (STAT1). Subsequently, this will lead to upregulation of antigen presenting MHC-I molecules on the beta-cell surface and secretion of chemokines, such as chemokine (C-C motif) ligand 3 (CCL3), CCL4 and C-X-C motif chemokine ligand 10 (CXCL10), which are able to attract more innate and adaptive immune cells to the islets [7,8]. Prolonged exposure of beta-cells to inflammation is known to induce oxidative and endoplasmic reticulum (ER) stress, associated with translational infidelity, alternative splicing, protein misfolding and the generation of post-translational modifications (PTMs) of proteins [9-11]. The unfolded protein response (UPR) will be activated, but at a point where this UPR fails to recover the cells from ER stress, apoptosis will be induced [12]. Such apoptotic beta-cells are cleared by antigen presenting cells (APCs), like macrophages and dendritic cells (DCs), which present beta-cell antigen-derived peptides on their cell surface in and the context of MHC-II molecules. In addition to peptides processed in these APCs, naturally processed beta-cell peptides, present in the ER due to degradation of proteins, will also be presented in the context of MHC-I on the beta-cells themselves [13].

When the beta-cell antigens in MHC-II molecules of APC are recognized by CD4+ T-cells, they will become activated effector T-cells which in turn stimulate the maturation of CD8+ T-cells

into cytotoxic T-cells by production of pro-inflammatory cytokines such as interleukin 2 (IL-2). These cytotoxic T-cells can recognize beta-cell antigens presented by MHC-I molecules on the beta-cell surface, resulting in granzyme- and perforin-mediated beta-cell death [14]. During the development of insulitis, beta-cells and immune cells are engaged in a dialogue. Activated T-cells, macrophages and Natural Killer (NK) cells will produce cytokines such as IL-1β, IFN-γ and tumor necrosis factor α (TNFα) and beta-cells respond by producing more chemokines and stimulatory cytokines. If at this point the viscous circle is not halted, the road is open to the development of T1D [15].

Proteomic techniques and studies have contributed to a large extend to our understanding of T1D development leading to the above-described hypothesis, but also to the characterization of novel biomarkers and therapeutic applications. In this review we highlight the important role of proteomics to identify beta-cell targets as novel biomarkers and therapeutic applications in T1D. An overview is given on recent proteomic studies that further explored the active role of the beta-cell in the pathogenesis of T1D, as well as new approaches that are developing.

2. Proteomics: the key towards a cure for T1D

When the total functional beta-cell mass is reduced to 20-30%, hyperglycemia and associated glycosuria will arise, leading to the onset of overt diabetes. Nowadays, diagnosis is based on elevated random plasma glucose levels of >200 mg/dl, a fasting blood glucose of >126 mg/dl or glycated hemoglobin A1c (HbA1c) levels of >6.5%. At this point lifelong treatment by exogenous insulin administration is initiated in most of the patients. To enable the identification of individuals who are susceptible to T1D development at an early stage, screening tests can be performed. The strongest genetic predisposition for T1D is associated with certain HLA genes [16-18]. As such, 90% of the patients are carriers of either HLA-DRB1*03 – DQB1*0201 (DR3 – DQ2) or DRB1*04 – DQB1*0302 (DR4 – DQ8) and 30% of them are even heterozygous for both (DR3/4) [19]. Compared to this, the prevalence in the general population is only 30%. Screening for this strong genetic risk factor indicates people with increases risk for T1D or other autoimmune diseases, but the sensitivity and specificity remain rather low. Therefore, the presence of autoantibodies in the circulation against beta-cell antigens such as insulin, glutamic acid decarboxylase of 65kDa (GAD65), tyrosine

phosphatase-like protein ICA152 (IA-2) and zinc transporter 8 (ZnT8) are used as more pathologically relevant targets to screen for in predisposed individuals. These antibodies are a consequence of the autoimmune reaction rather than being pathogenic themselves. However, during the period of progressive beta-cell destruction, in which normal blood glucose levels are still retained, autoantibodies already arise in the circulation indicating an ongoing autoimmune attack [20-24]. The number of different autoantibodies detectable is in direct relation to the relative risk to develop T1D in the future [20-22]. Whereas antibody based screening tests are much more predictive compared to genetic screening, the sensitivity still remains rather low, in particular in the general population.

Several hurdles still exist on the path to the ultimate goal, namely preventing or finding an effective cure for T1D. First of all, there is a need for better biomarkers with improved sensitivity and specificity, to be able to identify people progressing towards diabetes at an early stage, when the functional beta-cell mass is still high. Second, we must be aware that T1D is a heterogeneous disease, as evidenced by for instance the wide spread in age at disease onset, differences in genetic susceptibility factors and the diversity in serum antibodies against beta-cell antigens between all patients. In addition, recently described stress-induced PTMs of beta-cell antigens occur and elicit auto reactivity only in a subset of patients. This diversity indicates that patients with T1D might have different underlying pathogeneses. Finding a good combination of biomarkers, which reflects the underlying pathogenesis of the disease, is of utmost importance to be able to stratify patients and develop more personalized therapies.

3. Different proteomic approaches: strengths and limits

Over the years, proteomic approaches have developed rapidly resulting in an increasing sensitivity, specificity and applicability on smaller samples. Depending on the experimental goal and sample type, multiple approaches can be considered, each with their own pros and cons. In the past, many studies have used the gel based 2-dimension difference gel electrophoresis (2D-DIGE), which has the advantage to investigate quantitative expression levels in a robust and rather simple way. However, relatively high amounts of protein are required, making this method inappropriate for the analysis of scarce samples. Also the fact that a certain pH range is analyzed implies that only a fraction of proteins are analyzed.

Furthermore, reproducibility is rather weak and since protein levels are investigated by fluorescent labeling, low abundant proteins are likely to be under the detection limit. Due to these limitations, mass spectrometry based techniques gain in popularity. In general, a distinction is made between top-down approaches, investigating whole proteins, and bottom-up approaches where peptides after protein digestion are analyzed. In both cases, mass-to-charge ratios (m/z) are determined and used for protein identification by database searches. Prior to mass analysis, samples undergo ionization, with matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) being the main techniques. MALDI is a fast ionization technique, often used for samples with a low complexity. No prior separation step is required, opposed to for instance proteins extracted from 2D-gel spots. The ESI technique is used more commonly for ionization of complex samples, since this technique can easily be combined with liquid chromatography (LC) separation. Either ionization techniques were originally combined with time-of-flight (TOF) or TOF-TOF instruments. However, nowadays ESI is often used in combination with Orbitrap mass analyzers, which have significantly improved mass resolution and sensitivity [25,26]. Evidently, this goes along with an enormous increase in the amount of data generated, which requires suitable bioinformatics approaches to get interpretable knowledge out of these big-data lists.

4. Why is the beta-cell attacked by the immune system in T1D?

4.1. The beta-cell proteome in T1D

In the early years of proteomic research in the field of diabetes, many investigators aimed to establish reference maps of the pancreas, islet and beta-cell proteome to get a better insight in the general protein expression profile [15]. A strong representation of molecular chaperons and proteins implicated in metabolic pathways emphasizes the role of beta-cells as secretory cells which are specialized in producing enormous amounts of insulin. This also raised interest in studying more in depth the secretory granule proteome, to investigate which other proteins are secreted simultaneously with insulin [15]. Whereas it remains a challenge to isolate pure secretory granules, most of these studies indicate that next to the well described secretory granule proteins such as proinsulin, chromogranin A (ChgA), secretogranins and vesicle-associated membrane proteins (VAMPs), also proteins previously known for being functional in mitochondria, lysosomes or ER such as chaperons, are present [27]. Although

this might partially reflect incomplete cellular fractionation, there are also arguments that at least some of these proteins play an active role in the secretory granules [15]. As several described T1D autoantigens are present in the secretory granules, knowledge of this protein repertoire provides more information about other proteins potentially at risk to become antigenic when beta-cells are for instance under stress.

In order to investigate the islet and beta-cell proteome in the setting of T1D, ex-vivo exposure to IL-1β alone or in combination with other pro-inflammatory cytokines such as IFNγ and TNFα has been performed by many researchers. A synergistic action of these cytokines was observed, with major alterations in the beta-cell proteome, paralleled by induction of apoptosis and ER stress, as opposed to minor changes upon exposure to IL-1β alone. Recently it was described that human islets tend to be more resistant to cytokine-induced ER and oxidative stress as compared to rodent islets, and that TNF-α plays a more prominent role in the human setting [28].

Although many of these studies had the same goal, namely to gain a better insight in beta-cell function and dysfunction in T1D, by means of similar experiments, the results were often very different as displayed by the number of differentially expressed proteins as well as the protein identity or characterized pathways. This makes it very difficult to assign specific alterations to the effect of inflammation. An obvious explanation for the observed discrepancies is the fact that islets from different species, exposed to a slightly different combination and concentration of cytokines, were used. Moreover, the implication of distinct proteomic techniques and sample preparation methods should also not be underestimated.

4.2. New insights in the beta-cell proteome in T1D

A detailed overview of all proteomics studies in the field of T1D until early 2015 have been described in a previous review by our group [15]. In follow-up of this, we focus here on the most recent advances and novel technical approaches [Table 1].

Although mostly preference is given to human islets, animal models are still indispensible for gaining mechanistic insights. To answer one of the main questions namely ‘Why is the beta-cell attacked by the immune system in T1D?’ several studies aimed to identify predisposing genes and proteins in islets from pre-diabetic mice. Whereas the majority of these studies

focused on the role of the immune system by investigating islets from normoglycemic mice with already ongoing immune destruction [29-32], our group recently investigated whether islets from 2-3 week-old diabetes-prone NOD mice, i.e. before the occurrence of immune infiltration, differ from control mice, using a combination of 2D-DIGE and microarray. The most remarkable difference was the observation that NOD islet proteins are undergoing different PTMs, in particular citrullination by peptidyl arginine deiminase 2 (PAD2), compared to control mice. In addition, lower expression levels of proteins involved in correct folding, such as Protein disulfide isomerases (PDIA) 3, 4 and 6 make NOD islets more sensitive for protein misfolding, potentially leading to ER stress [33]. A 2D-DIGE-proteome comparison of NIT-1 beta-cells and αTC-1 alpha-cells confirmed that specifically the beta-cells are highly susceptible to ER and oxidative stress upon inflammation [34]. To clarify further downstream cytokine-mediated pathways, Rondas et al. investigated islets from STAT-1 knock-out (KO) mice by 2D-DIGE. These islets were mainly impaired in protein synthesis and processing and based on network analysis, small ubiquitin-related modifier 4 (SUMO4) was hypothesized as playing a central role in STAT-1 signaling [35].

In order to investigate a specific pancreatic T1D signature, Burch et al. performed comparative proteome analysis from snap frozen human pancreatic tissue lysates from donors with T1D, T2D, autoantibody positive non-diabetic and autoantibody negative non-diabetic donors using LC-MS/MS (quadrupole-Orbitrap). In total 1149 proteins were identified of which 244 proteins were differentially expressed, with 134 upregulated and 110 downregulated, in T1D compared to the non-diabetic donors. Functionally, these proteins were shown to be mainly involved in inflammation, metabolic regulation, and autoimmunity [36].

The procedure to isolate pancreatic islets is associated with a certain amount of stress as such, which can result in proteomic alterations by itself. New techniques have been explored over the last years to overcome this drawback. Laser capture micro dissection (LCM) allows the isolation of specific type of cells from a tissue, such as the islets out of a pancreatic section. Recently, Zhang et al. [37] used this approach combined with nano LC-MS/MS to compare the proteomic profile of human islets and acinar tissue. This led to the identification of 1104 and 706 proteins, respectively. A high proteomic overlap was observed between LCM islets and enzymatically isolated islets (984 proteins), but interestingly less stress-associated proteins and acinar proteins were identified using the first method. The relatively large proteome

coverage, together with good reproducibility and technical feasibility underscores the importance of LCM to become a more routinely used technique in future studies.

MALDI imaging mass spectrometry (IMS) allows the highest possible cellular resolution achievable in shotgun proteomics nowadays. The minimal distortion of histological features during analysis allows a better understanding of biological processes [38]. In 2011, Green-Mitchell et al. [39] used MALDI-IMS to identify a peak of 5812.85 Da, being highly expressed in islets from healthy subjects compared to T1D patients. *In-situ* reduction followed by alkylation was needed to allow identification of this protein peak as insulin. Since technical advances are developing rapidly, it is today already possible to detect higher molecular weight proteins up to 20 kDa using this sophisticated technology.

Immunopeptidome analyses of beta-cells are performed in order to map the peptide repertoire presented in surface MHC-I molecules. These epitopes might be highly important for initiation or progression of autoimmunity since they are exposed to cytotoxic T-cells [40]. When investigating alterations in the peptidome of NIT-1 beta-cells upon exposure to IFNγ, Dudek et al. did not observe differences in peptide length or distribution of predicted binding affinities. However, significantly higher presentation of the immunodominant epitope of IGRP (206-214) upon cytokine exposure compared to a random endogenous peptide, was identified by multiple-reaction monitoring (MRM). On the other hand, the peptidome presented by immune cells is also important to gain a better understanding of the autoimmune process. Van Lummel et al. investigated the peptidome presented by immune cells, by pulsing dendritic cells with preproinsulin, GAD65 and IA-2. Subsequent analysis revealed that GAD65 peptides from previously identified immunogenic epitopes could be presented in HLA-DR3. For IA-2, previously unknown immunogenic peptides from the extracellular domain were found to be presented in HLA-DR3 and DR4 and they elicited T-cell responses in T1D patients [41,42]. In addition, antigenic PPI and IA-2 were mainly presented in HLA-DQ8trans molecules, explaining the genetic risk of HLA-DQ2/8 heterozygosity [43]. More extensive studies are required to unravel the complex mechanism of peptide presentation, but it is presumed that stress-induced PTMs, in combination with genetic susceptibility, are also involved [44]. In general, peptidome studies contributed substantially to a better definition of consensus sequences for antigens presented in HLA molecules. This information has been implied in predictive bioinformatics approaches [40]. However, one

must keep in mind that immunogenic epitopes in autoimmunity are thought to have rather weak binding affinity for HLA molecules [45,46]. More extensive comparison of the beta-cell and thymus peptidome would be interesting to gain insight in the underlying causes of beta-cell targeted autoimmunity [40,47]. Although no major general differences have been observed, a more directed approach to study some peptides in particular would be informative. Whereas, analysis of the immunopeptidome is also technically challenging, Caron et al. reviewed nicely the differences and advantages of several currently used MS/MS approaches [48].

Recent studies have led to the hypothesis that T1D does not only involve the beta-cells in the pancreas. In addition to glucagon producing alpha-cells, several studies even reported abnormalities in function, as well as histology and anatomy, of the exocrine pancreas of T1D patients [49]. Proteomic analysis by tandem mass tag (TMT) and multidimensional LC-MS/MS of the exocrine pancreas revealed that 145 proteins of a total of 5357 proteins were differentially expressed in the exocrine pancreas of T1D patients compared to healthy subjects. These proteins were functionally related to the ubiquitin proteasome system, cell proliferation and apoptosis. Whereas this suggests the involvement of exocrine pancreatic cells in the pathogenesis of T1D, further investigation is needed to reveal if such findings are rather the consequence or cause of autoimmunity [50].

4.3. Stress induced PTMs of beta-cell antigens: mechanism for break of tolerance

It is estimated that 50-90% of all proteins in the human body are modified post-translationally and the majority of them are present in multiple isoforms. Modification can occur either by the attachment of chemical molecules to amino acids or by changing the chemical bond structure of proteins. Under physiological conditions, the main function of PTMs is the control of protein function, such as enzymatic activation, subcellular localization, stability, structure and interaction with other proteins or cells. While the total time needed for transcription and translation can take several hours, PTMs are able to activate or inactivate specific protein functions in a very rapid and dynamic way. Apart from this, activation of specific modifying enzymes as a result of cellular stress, such as tissue transglutaminases (tTG) and PADs, can lead to the generation of neo-epitopes. It is known from several autoimmune diseases, including T1D that such modifications can lead to a break of immune tolerance [Table 2].

Several mechanisms can be responsible for this. At the peptide level, PTMs can modulate the affinity and positioning of peptides in the HLA binding groove. As such, it is known that certain diabetes susceptible HLA types preferentially present modified peptides, such as HLA-DR4 and DR3 for citrullinated peptides and HLA-DQ2 and DQ8 for deamidated peptides [44]. At the protein level, PTMs might influence processing by proteases, resulting in an altered pool of peptides generated in stressed tissues such as the islets in T1D, as compared to the thymus. In addition, PTMs that affect the tertiary structure can result in exposure of internal epitopes at the outside of the protein, which could potentially lead to B-cell receptor recognition.

The first modified antigen in T1D was described in 2005 by Mannering et al. They demonstrated a disulfide bond between cysteine A6 and A7 of a naturally processed peptide of the insulin A-chain (A1-13). This modification had no effect on the presentation by HLA-DR4, but was shown to be crucial for recognition by CD4+ T-cell clones from a T1D donor and an insulin autoantibody positive donor at risk for T1D [51].

More recently, Stadinski et al. [52] identified WE14, a naturally processed peptide from the secretory granule protein ChgA, as target antigen for diabetic T-cell clones in NOD mice. Although the WE14 peptide contains the WXRM amino acid motif, a mimotope sequence for activation of these T-cell clones, very high concentrations of the peptide were needed *in vitro* to activate T-cells. Moreover, the WE14 peptide could not be detected by MS in antigenic fractions, opposed to the detection of full length ChgA. Of interest, PTM of WE14 by enzymatic treatment with tTG resulted in increased stimulation of diabetic T-cell clones [53]. Separation of *in vitro* transglutaminated WE14 peptide on SDS-PAGE showed that aggregates were formed upon tTG exposure and fractionation by size exclusion chromatography, followed by co-culture of these fractions with diabetogenic T-cell clones revealed that both high and low molecular weight aggregates were the actual inducers of enhanced antigenicity. In follow-up of this study in mice, it was shown in humans that peripheral blood mononuclear cells (PBMCs) of recent onset T1D patients recognized WE14 in a dose dependent way and similar as for NOD T-cell clones, TGase treatment increased the T-cell response [54]. Furthermore, the pathogenic importance of ChgA as autoantigen was proven by protection of NOD mice deficient in ChgA against diabetes development [55]. In the same line, Van Lummel et al. [56] discovered that tTG modification of other T1D antigens enhanced their antigenicity, suggesting a more general role for this modification in T1D. They screened

known T1D antigen peptides with the tTG substrate motif (e.g. proinsulin, ZnT8, IA-2, GAD65, ICA69, phogrin and IGRP) for binding to the T1D-predisposing HLA-DQ8cis/trans molecule. Deamidation of 28 peptides was confirmed, but activated T-cells could only be detected against a deamidated peptide of proinsulin. Furthermore, recent onset T1D patients showed much higher levels of autoreactive T-cells recognizing this modified proinsulin peptide as compared to healthy controls, confirming its role as a pathogenic autoantigen.

Marré et al. investigated the mechanism behind the presence of deamidated antigens in T1D and pointed to the importance of ER stress [44]. Exposure of mouse islets to chemical ER stress by thapsigargin (Tg) increased cytoplasmic Ca^{2+} levels, paralleled by elevated TGase activity and strong activation of the CD4+ diabetogenic BDC2.5 T-cell clone. The higher IFN-γ secretion by the T-cell clone against *in vitro* transglutaminated antigens further confirmed these findings. In addition to transglutamination, McGinty et al. showed the presence of antigenic GAD65 peptides modified by citrullination. Both modifications result in enhanced peptide binding to HLA-DR4 *in vitro* and recognition of these modified peptides by specific T-cell clones of T1D patients. Furthermore, the presence of significantly higher amounts of these T-cells was observed in T1D patients compared to HLA-matched healthy controls [57]. Our group discovered that glucose-regulated protein 78 (GRP78) becomes citrullinated in INS-1E beta-cells and mouse islets upon exposure to inflammatory stress. In addition, cytokines induced translocation of this molecular chaperon from the ER to the cell surface. As a result, citrullinated GRP78 was recognized as autoantigen in NOD mice, evidenced by the presence of autoantibodies and T-cell reactivity [58]. Furthermore, a very high expression of the citrullinating enzyme PAD2 was found in NOD islets compared to islets from control mice, further emphasizing the relevance of this gene for being the diabetes susceptibility gene in the *Idd25* susceptibility locus [33,58,59].

Next to these non-enzymatic and enzymatic modifications, Delong et al. described recently a novel mechanism of protein modification leading to a break of tolerance, namely the generation of hybrid insulin peptides (HIPs) [60,61]. They showed that CD4+ T-cell clones from NOD mice, but also CD4+ T-cells isolated from islets of T1D patients, show auto-reactivity against epitopes that were generated by covalent cross-linking of proinsulin peptides with other secretory granule peptides such as WE14 an IAPP. This kind of PTM, novel in autoimmune diseases, was already described as a mechanism for tumor antigen formation.

In this case, peptide hybrids are formed by trans-peptidation during proteasomal degradation [62-64]. In beta-cells, HIPs are presumed to be generated during proteolytic hydrolysis in secretory granules, where also naturally occurring cleavage products such as WE14 are formed [65].

Whereas T-cell responses are mainly investigated in peripheral blood, Babon et al. studied the repertoire of islet-infiltrated T-cells. CD4+ and CD8+ T-cell lines and clones were generated out of infiltrated islets from T1D patients and several of these clones reacted against a broad range of native beta-cell antigens. In addition, reactivity was also detected against PTM proteins such as citrullinated GRP78, citrullinated IAPP and HIPs, giving even more evidence for the importance of PTMs in loss of tolerance against beta-cell antigens [66].

Kracht et al. recently discovered the expression of a defective ribosomal gene product (DRiP) of insulin, resulting from translation of an alternative open reading frame. Inflammatory and ER stress lead to increased expression levels of this DRiP protein. DRiP generation has been previously described in cancer cells, but is a novel mechanism for autoantigen generation in T1D. Autoreactive CD8+ T-cells that recognize this out-of-frame translational product of insulin were detected in blood of T1D patients and furthermore, these cells were capable of killing human beta-cells *in vitro* [67].

PTMs of beta-cell antigens are not only involved in activation of the cellular immune system. Patients with T1D also have higher levels of serum autoantibodies against modified epitopes, such as oxidized GAD65 [68,69], hydroxyl radical modified GAD65 [70], oxidized insulin [63,71], oxidized collagen II [72] and phosphorylated peripherin [73].

To conclude, the important role that PTMs or alternative ribosomal products play in the pathogenesis of T1D became clear during the last 10 years. Since pro-insulin is the only beta-cell specific antigen in T1D, it remained for long a mystery how autoimmunity against ubiquitously expressed proteins could lead to a specific attack of the beta-cells. The generation of PTMs under stress conditions in beta-cells, inducing structural changes of ubiquitously expressed proteins specifically in the beta-cell, can provide an explanation for this phenomenon.

5. How to see beta-cell autoimmunity? The need for novel biomarkers

A high number of novel autoantigens or mechanisms implicated in the pathogenesis of T1D have been discovered during the last years. However, a major issue in the design of effective prevention and intervention studies is the lack of precise biomarkers to detect early stage of beta-cell decomposition or to use as surrogate endpoint in clinical studies. In 1998, the National Institutes of Health defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [74]. Importantly, biomarkers should be measurable in easily available body fluids such as blood, serum, plasma, urine or saliva. We refer to a previous review by our group for a detailed overview of serological biomarkers in T1D [15] and will focus here in particular on recent advances in the field.

In most biomarker studies serum proteome analysis was performed. This is a challenging approach because of the wide dynamic range in abundance of proteins as well as the presence of high abundant serum proteins, such as albumin, transferrin and fibrinogen, all together accounting for 95% of the serum proteome. To overcome these issues, serum samples are often pre-processed, for instance by using hexa-peptide library beads or strong cation exchange (SCX) columns, to deplete these high abundant and at the same time enrich for the low abundant proteins [64]. Subsequent proteome analysis mainly includes shotgun proteomic techniques such as LC-MS/MS.

Metz et al. [75] identified 24 serum proteins with altered expression in T1D patients that were associated with innate immunity, inflammatory response, blood coagulation and complement system. Later on, Zhang et al. [76] reported the validation of this study in samples from children aged 5-15 years using the LC-MS/MS accurate mass and time tag (AMT) method with selected reaction monitoring (SRM) [77]. Furthermore, this study showed that plasma protease C1 inhibitor and platelet basic protein achieved absolute sensitivity and specificity for classification of T1D samples.

Although serum levels of proteins secreted by beta-cells are expected to be expressed at very low levels, Pepaj et al. succeeded in identifying transmembrane protein 27 (TMEM27), and showed a lower expression of this protein in serum from T1D patients compared to healthy controls. Furthermore, they showed by SILAC combined with LC-MS that that this beta-cell protein, important for insulin secretion and beta-cell proliferation, was highly upregulated in

INS-1 cells upon 1,25dihydroxyvitamin D3 treatment [78]. Similarly, serum levels of insulin-like growth factor 1 (IGF1) or IGF binding protein 3 (IGFBP3) were correlated to seroconversion and disease onset in genetically at risk children for T1D [79].

Aiming to predict progression to T1D by measuring a minimal number of serum peptides, Von Toerne et al. compared the serum proteome of islet autoantibody positive and negative children from the BABYDIAB/BABYDIET cohort. A significant enrichment of proteins involved in lipid metabolism was highlighted. Two peptides in particular, from apolipoprotein M and apolipoprotein C-IV, made it even possible to distinguish both groups [80]. Furthermore, Manjunatha et al. described that T1D patients have higher levels of irreversibly modified HDL proteins, such as amadori modification of ApoD and deamidation, compared to healthy subjects [81]. These modifications could explain the compromised function of HDL and the associated increased cardiovascular risk in T1D patients, however no difference was observed between patients with a good or poor glycemic control, suggesting that measuring serum levels of modified lipoproteins cannot be used as biomarker for cardiovascular risk in T1D patients.

In addition to a broad MS analysis of the serum proteome, immunoproteomics is a more directed approach to evaluate ongoing autoimmunity against beta-cell antigens, by detecting autoantibodies in serum of T1D patients. One of the first studies was reported by Massa et al., who performed serological Proteome Analysis (SERPA) by separating beta-cell proteins on a 2D gel followed by blotting on a membrane and subsequent detection with serum of T1D patients who were positive against only one known beta-cell antigen [82]. Although several proteins such as carbonic anhydrase, PDI and tubulins were proposed as possible autoantigens, this has not been confirmed until now.

Most recent studies make use of protein arrays, where immunoreactivity of serum is tested against a large number of proteins. LaBaer and Ramachandran developed a Nucleic Acid Programmable Protein Array (NAPPA) by spotting cDNA clones on a slide, leading to protein synthesis and capture [83]. Miersch et al. [84] used NAPPA to identify serum autoantibodies against 6,000 human proteins. They reported reactivity against 27 proteins in T1D patients, including ZnT8 and 26 novel antigens. Further, validation with Luciferase Immuno-Precipitation System (LIPS) assay verified the presence of autoantibodies to dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) with 36% sensitivity at 98% specificity.

More recently, Bian et al. screened for the presence of autoantibodies against 10,000 human proteins, which led to the identification and validation of six novel T1D associated autoantibodies, against PTPRN2, MLH1, MTIF3, PPIL2, NUP50 and QRFPR, having a sensitivity of 16-27% and specificity of 95% [85]. In addition, they aimed to evaluate the association between T1D and viral infections by making use of customized arrays comprising 646 viral antigens. This indicated that T1D patients significantly recognized Epstein-Barr viral peptides, whereas previously mainly cockxackie viruses were attributed as being involved in T1D pathogenesis [72].

Whereas most serum biomarkers are indicative for beta-cell autoimmunity, biomarkers that display evolving complications can be useful as well. Several proteomic analyses on urine samples of T1D and T2D patients have been performed in order to identify biomarkers that predict diabetic nephropathy. Most of these studies were performed by LC-MS/MS, which is known to have better peptide resolution and accuracy rates compared to MALDI-MS [86]. Caseiro et al. performed a proteome study by LC-MS/MS on urine of T1D patients with and without complications. Increased urinary levels of gelsolin and antithrombin-III were observed in all T1D patients, while ephrin type-B receptor 4 and vitamin K-dependent protein Z were identified as promising biomarkers for both retinopathy and nephropathy [87]. Suh et al. identified 1036 protein in urine samples by nano LC-MS/MS, with 50 of them showing a significant difference between T1D and healthy people. A distinctive observation was the high abundance of 13 lysosomal proteins and 15 proteins involved in vascular permeability and adhesion, pointing to hyperglycemia-associated inflammation in the kidney vasculature [88]. Although these studies are promising, further research is needed to reveal the clinical applicability of these proteins as biomarker. Meanwhile, the detection of albuminuria remains the golden standard to evaluate the development of nephropathy.

6. Conclusion

The search for a successful preventive strategy or an effective therapy to cure T1D engages the combined efforts of many scientists from different disciplines nowadays. Due to the multifactorial nature of this disease, collaboration and pooling of knowledge between different research domains such as immunology, endocrinology and molecular biology will be required. Proteomics plays a central role in this network, as an important tool to gain more

insight in the underlying pathogenesis of T1D by unraveling pathways associated with beta-cell dysfunction. Furthermore, novel autoantigens or mechanisms to generate antigenicity, such as PTMs, HIPs and DRiPs, were discovered with the help of proteomic techniques. These will be important for further exploration as therapeutic targets. While this review focused on proteomics, the search for biomarkers is not restricted to this research field only. During recent years, many promising advances were made also in the identification of circulating microRNAs [89] and metabolomics [90]. In order to achieve sensitive, robust and personalized screening tests to predict the onset of T1D, therapeutic efficacy or the development of complications, it will be of utmost importance to combine knowledge from the fields of genetics, transcriptomics, proteomics and metabolomics. Advanced Systems Biology is expected to become of crucial importance in such meta-analyses.

7. Expert commentary

During the last 15 years, the importance of PTMs as a mechanism to induce a break of tolerance against self-antigens, became clear in many autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, colitis, multiple sclerosis and also type 1 diabetes (T1D) [91]. Whereas in the past autoimmune disorders were rather considered as distinct diseases, over the years more and more evidence have pointed to common underlying pathogenic pathways. This is in particular the case for the generation of PTMs upon exposure of tissues or cells to inflammatory, ER or oxidative stress in different autoimmune diseases. The enzymes required for deamidation and citrullination, namely tTG and PAD, require high Ca^{2+} levels, which are reached under such stress situations [44]. But also HIPs [60] and DRiPs [67] are more abundantly identified under these circumstances. This concept may also explain why ubiquitously expressed proteins can be recognized as autoantigens, thereby leading to an autoimmune attack in a specifc target tissue, while other organs remain protected or unaffected. Furthermore, the observed differences in immune responses against modified beta-cell antigens between different patients, may be one of the important explanations for the heterogeneity of T1D. To our understanding, the new discoveries of the last years on newly described unconventional beta-cell antigens are probably just a tip of the iceberg.

Therefore, biomarkers that allow mapping of these differences in the underlying pathogenesis of T1D would be of utmost importance to stratify patients according to these characteristics. Furthermore, this would also be a starting point to approach therapies in a personalized way, for example by targeting tTG or PAD enzymes in patients where deamidation or citrullination were shown to be implicated in disease onset or progression. Although there is still a long way to go, step-by-step T1D research is bringing us closer to the development of personalized medicine. However, with current technologies, some limitations are still being faced that impede high throughput identification of more unconventional proteins or peptides. First of all, PTMs such as deamidation and citrullination result in a very small difference of only 0.984 Da in molecular weight compared to the native peptides. Even for the most sensitive mass analyzers, this difference is at the limit of detection. In addition, the prevalence of these modified peptides are typically extremely low abundant. At present the majority will probably not be identified or have such low confidence scores that they will be missed during analysis. Even though it is important that scores are considered strictly, it should be advised to be thoughtful when analyzing such data. Besides PTMs that occur by chemical amino acid modification, the difficulty to discover new HIPs and DRiPs is even more challenging since these sequences are unknown and currently not present in any database. As it is known that after MS/MS analysis, up to 50% of the peptides with a good score do not result in a satisfying identification, there is a reasonable chance that HIPs, DRiPs or even other alternative peptides are present and missed in current routine MS analyses. As nowadays, characterization of these peptides is only possible by a directed search for a specific peptide sequence, advances in mass spectrometry, development of new bioinformaticsespecially also in the search programs that would allow picking up such unconventionally processed or PTM modified proteins are warranted. This would mean a big step forward in unraveling the dark side of the proteome.

8. Five year View

Using human tissue is the desired path in proteome research of human diseases and is occurring more frequently in recent years. However, it still remains challenging in the field of T1D because of the limited availability of human islets. The development of highly sensitive techniques that only require small samples for analysis, major technical improvements in

detection tools and more efficient sample preparation, will lead to an increased use of human islet samples for research purposes in the coming years. In order to optimally utilize these precious samples, making them available for multiple studies and sharing and combining data more efficiently, several consortia have been established [92]. For example, the biobank "Network for Pancreatic Organ Donors with Diabetes" (nPOD) (http://www.jdrfnpod.org), launched in 2006 and supported by Juvenile Diabetes Research Foundation (JDRF), has the goal to distribute pancreases and other tissues from T1D autoantibody positive individuals [93]. Recently, INNODIA, a European initiative was launched, funded by the Innovative Medicines Initiative – Joint Undertaking (IMI-JU), involving academic and industry partners as well as foundations (JDRF and The Leona M. and Harry B. Helmsley charitable trust) (www.INNODIA.eu). This large network, also involving patients and their family members, is collecting samples from newly diagnosed T1D patients and first-degree relatives and is investigating novel biomarkers for disease progression.

While until today, proteomics approaches are mainly applied in T1D fundamental and pre-clinical research, the importance in the diagnostic clinical field is expected to grow. It remains challenging to obtain high quality protein samples from blood, but improved purification strategies are continuously being optimized. Recently, multiple biomarkers that display the progressing development of T1D or associated complications were identified, of which the prognostic value is currently further confirmed. Remarkably, only a subpopulation of patients turns out to test positive for a specific biomarker. This further demonstrates the importance of personalized screening and diagnostic tests in order to apply suitable therapies to patients.

Key Issues

- Proteome profiling of islets, blood samples or other body fluids from T1D patients provides crucial information.
- In addition to cell-lines, isolated islets of Langerhans from rats, mice and humans are widely used as a more physiological beta-cell source. A number of consortia and cohorts are launched to provide research samples such as islets, blood and serum from healthy and diabetic patients to researchers.

- Several studies used proteomics approaches to establish reference maps of the pancreas, islet and beta-cell proteome to get insight in the general expression profile.
- The use of MALDI-IMS, LCM-based proteomics and immunopeptidome approaches in T1D studies helped in the identification of novel antigens.
- The role of PTMs in the generation of neo-epitopes in T1D is highlighted. Also autoreactive T-cell responses towards HIPs and DRiPs showed the importance of unconventionally processed peptides in autoimmune diseases such as T1D.

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Declaration of interest

The authors have declared no conflict of interest.

Table 1. Overview of publications on beta-cell proteome and immunopeptidome studies

Author	Species	Cell type	Proteomics Technique	Study Type	Year	Reference
Crèvecoeur et al.	Mus musculus	Islets	2D-DIGE,MALDI-TOF/TOF	Differential expression	2017	[33]
Gorasia et al.	Mus musculus	Islets and NIT-1 and αTC-1	MALDI-TOF/TOF	Differential expression	2015	[34]
Rondas et al.	Mus musculus	Islets	MALDI-TOF/TOF	Differential expression	2015	[35]
Burch et al.	Homo sapiens	Pancreas	LC-MS/MS	Reference Map	2015	[36]
Zhang et al.	Homo sapiens	Pancreas	LC-MS/MS	Reference Map	2017	[37]
Green-Mitchell et al.	Homo sapiens	Pancreas	MALDI-MSI	Reference Map	2011	[39]
Dudek et al	Mus musculus	NIT-1	LC-MS/MS	Immunopeptidome	2012	[40]
van Lummel et al.	Homo sapiens	PBMC	FT-MS	Immunopeptidome	2016	[41]
Peakman et al.	Homo sapiens	PBMC	RP-HPLC, MALDI-TOF-MS	Immunopeptidome	1999	[42]
van Lummel et al.	Homo sapiens	PBMC	FT-MS	Immunopeptidome	2016	[43]
Espinosa et al.	Homo sapiens	Thymus	LC-MS/MS	Immunopeptidome	2013	[47]

Table 2. Overview of publications on PTMs, HIPs and DRiPs in T1D

Modification Type	Protein/Peptide	Year	Author	Reference
Disulfide bond	Insulin A chain	2005	Mannering et al.	[51]
Transglutamination	WE14 Peptide of ChgA	2012	Delong et al.	[53]
Deamidation	Proinsulin	2014	van Lummel et al.	[56]
Citrullination and Transglutamination	GAD65	2014	McGinty et al.	[57]
Citrullination	GRP78	2015	Rondas et al.	[58]
Citrullination and HIPs	GRP78, IAPP	2016	Babon et al.	[66]
HIPs	insulin-IAPP hybrid	2016	Wiles et al., Delong et al.	[60, 61]
DRIPs	Insulin	2017	Kracht et al.	[67]